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THE OVARIAN INSULIN AND INSULIN-LIKE GROWTH FACTOR SYSTEM WITH AN EMPHASIS ON DOMESTIC ANIMALS[1]

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ABSTRACT

Insulin and insulin-like growth factors (IGFs) have direct effects on cultured ovarian cells. These effects include stimulation of granulosa cell mitogenesis, granulosa and luteal cell progesterone production, and thecal cell androgen production and appear similar among species. However, species differences exist with regard to insulin and IGF-I effects on granulosa cell estradiol production. In addition to endocrine effects of insulin and IGFs, IGFs are produced by granulosa, thecal, and luteal cells, allowing for an intraovarian autocrine and paracrine system. Granulosa, thecal, and luteal cells contain receptors for insulin and IGFs, and these receptors appear to mediate the effects of insulin and IGFs. Adding to the complexity of the regulatory role of IGFs is the presence of IGF-binding proteins (IGFBPs) within the ovary. These IGFBPs are produced by granulosa, thecal, and luteal cells, and their production is hormonally regulated. Evidence for a coherent mechanism by which insulin, IGFs, and IGFBPs interact and regulate ovarian function in vivo has yet to be found.

INTRODUCTION

Over 20 years after the determination of the primary amino acid sequence of insulin in 1955 (1), a role for insulin as a regulator of ovarian function was suggested (2). It took an additional 10 years to determine the mechanism of insulin action on ovarian cell steroidogenesis as well as to determine the presence of insulin receptors in ovarian cells (for a review, see Ref. 3). In general, insulin's effects on ovarian cells are positive, stimulating granulosa cell proliferation and the production of progesterone and enhancing luteal cell steroidogenesis regardless of species. Various aspects of insulin's action on ovarian cells will be detailed below.

Related structurally and functionally to insulin, insulin-like growth factor-I (IGF-I) and IGF-II were purified and sequenced in 1978 (4,5). Approximately 45% of the amino acids comprising IGF-I, IGF-II, and insulin are identical; structural similarities across several species are compared in Table 1. Since that time, the presence and function of IGF-I and -II in many tissues of the body have been identified (for reviews, see Refs. 6–9). Among these is their presence and function in the ovary of many species (for reviews, see Refs. 10–13). For example, in cattle and pigs, IGF-I has been found to stimulate granulosa cell proliferation and mitogenesis (14–16) and enhance follicle-stimulating hormone (FSH)-induced steroidogenesis by granulosa cells (15–17). In addition, the presence of high-affinity, low-capacity binding sites for insulin and IGF-I in granulosa cells of pigs and

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TABLE 1. HOMOLOGY OF IGFS AND INSULIN AMONG SPECIES.[a]

Bovine Insulin/IGF	Ovine	Porcine	Human	Rat
IGF-I (%)	99	100	100	96
IGF-II (%)	99	97	96	96
Insulin (%)	≥97	≥95	≥93	≥95

[a] Compiled from several sources.

cattle has been documented (16–21). Moreover, the production of IGF-I by bovine and porcine granulosa cells appears to be under hormonal control (15,22,23). More recently, hormonally sensitive IGF-binding proteins (IGFBPs) have been found to be secreted by porcine granulosa cells in vitro (24–26). This review will summarize current evidence regarding the nature of the multiple components of the ovarian insulin and IGF system, emphasizing data generated in domestic animals.

ROLE OF INSULIN AND IGF IN OVARIAN FUNCTION

Granulosa cell mitogenesis. Studies in swine, sheep, and cattle have shown stimulatory effects of insulin and IGF-I on granulosa cell proliferation and(or) DNA synthesis (15,16,27–33) and are summarized in Table 2. The ED_{50} for the effects of insulin (100 ng/ml) and IGF-I (30 ng/ml) on mitogenesis indicates that IGF-I is a more potent mitogen than is insulin. In cultured porcine granulosa cells, IGF-I appears to enhance the mitogenic effects of other growth factors (29). In further support of a role for IGF-I in follicular growth, concentrations of IGF-I in the follicular fluid of cattle correlate positively with follicular diameter (see later section on "Ovarian Insulin and IGFs"). Few studies have evaluated the dose-response effect of IGF-II on granulosa cell proliferation, although IGF-II does stimulate DNA synthesis in cultured porcine (16,34) and human (35) granulosa cells. In rats, insulin and IGF-I also appear to stimulate granulosa cell mitosis (36–38) and this mitogenic effect of IGF-I is enhanced by FSH (38). Similarly, recent in vitro studies in cattle suggest that FSH and luteinizing hormone (LH) enhance the mitogenic effect of IGF-I in granulosa cells from small (<5 mm) but not large (<10 mm) follicles (33). However, FSH does not alter the stimulatory effect of IGF-I on the proliferation of ovine granulosa cells in vitro (31). In vivo, pregnant mare serum gonadotropin (PMSG)–stimulated prepubertal gilts treated with insulin have increased numbers

TABLE 2. SUMMARY OF THE MITOGENIC ACTION OF IGF-I IN BOVINE, OVINE, AND PORCINE OVARIAN CELLS.

Authors	Ref. No.	Year	Endpoint	Fold Increase	Cell Type	Species
Savion et al.	27	1981	Cell numbers	3	Granulosa	Bovine
Baranao and Hammond	16	1984	[^{3}H]thymidine[a]	5	Granulosa	Porcine
May et al.	29	1988	[^{3}H]thymidine	3	Granulosa	Porcine
			Cell numbers	3	Granulosa	Porcine
Kamada et al.	34	1992	[^{3}H]thymidine	3	Granulosa	Porcine
May et al.	46	1992	Cell numbers	2	Thecal	Porcine
Monniaux and	30	1992	[^{3}H]thymidine	2	Granulosa	Ovine
Pisselet			Cell numbers	2	Granulosa	Ovine
Zhang and	32	1993	[^{3}H]thymidine	3	Granulosa	Porcine
Bagnel			Cell numbers	5	Granulosa	Porcine
Chakravorty et al.	47	1993	[^{3}H]thymidine	4	Luteal	Bovine
Spicer et al.	15	1993	Cell numbers	2	Granulosa	Bovine
Monniaux et al.	31	1994	Cell numbers	2	Granulosa	Ovine

[a] [^{3}H]thymidine incorporation, a measure of DNA synthesis.

of small (but not medium or large) follicles (39), whereas increased numbers of small antral follicles are correlated with increased serum IGF-I in prepubertal gilts injected daily with porcine somatotropin (pST; 40). In prepubertal gilts treated with sustained-release implants of pST, increased numbers of medium follicles are correlated with increased serum IGF-I (41), suggesting that the mode of pST treatment may affect the size category of follicle that responds. In cattle, bovine ST (bST) treatment for two estrous cycles increased serum IGF-I concentrations and numbers of small (2 to 5 mm) follicles twofold without affecting serum LH or FSH or the numbers of large or medium follicles (42). Treatment with bST also increased serum IGF-I and the ovulation rate in superovulated beef heifers (43,44). However, insulin treatment (causing a twofold increase in plasma insulin concentrations) given concomitantly with FSH during the superovulation of cattle had no effect on the numbers of antral follicles of any size category (45). Collectively, in vitro and in vivo data support the notion that both insulin and IGF-I act as promoters of follicular growth, especially in pigs and cattle.

There is a paucity of information regarding the effects of insulin and IGFs on thecal and luteal cell proliferation. In cultured porcine thecal cells, IGF-I appears to enhance the mitogenic effects of other growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (46), but its effects are less pronounced than those observed with granulosa cells (29). Recently, Chakravorty et al. (47) reported that insulin was over 80-fold less potent than IGF-I in stimulating DNA synthesis (ED_{50} = 0.5 μg/ml vs. 6.0 ng/ml) in cultured bovine luteal cells and that their effects were not additive, suggesting a common mechanism of action. Clarification of the role of insulin and IGFs on thecal and luteal cell proliferation awaits further study.

Estradiol production. The effects of insulin, IGF-I, and IGF-II on estrogen production by granulosa cells seem to depend on the species studied. In rats and primates, studies indicate that insulin, IGF-I, and IGF-II can stimulate granulosa cell estradiol production in vitro (ED_{50} = 250 and 40 ng/ml for insulin and IGF-I, respectively), in a rank order potency suggestive of an effect mediated via type I IGF receptors (10–13,34,48–51). However, in cattle, insulin is a more potent and efficacious stimulator of estradiol production than is IGF-I (15,20,52,53). In fact, IGF-I at 100 ng/ml either had no effect or inhibited basal and FSH-induced estradiol production by bovine granulosa cells collected from small (i.e., 1 to 5 mm) follicles, whereas IGF-I at 100 ng/ml was a weak stimulator of FSH-induced estradiol production by granulosa cells from large (i.e., ≥8 mm) follicles (15,52). However, when granulosa cells were cultured in the presence of 1% fetal calf serum (which would contain insulin, IGF-I, and IGF-II), IGF-I and insulin had no effect on basal estradiol production by cells collected from large (12 to 15 mm) bovine follicles (54). More recently, we have observed that IGF-I and -II can inhibit insulin-stimulated estradiol production by granulosa cells of both small and large bovine follicles (20). We have further evaluated low-dose (i.e., ≤10 ng/ml) effects of insulin on bovine granulosa cell estradiol production and find that physiologic levels of insulin stimulate follicular estradiol production by more than threefold (Figure 1; 20). In support of these findings, we observed that, in vivo, insulin treatment (causing a twofold increase in plasma insulin concentrations) during the superovulation of cattle increased follicular fluid estradiol concentrations by fivefold in large follicles (45). Also in cattle, we have observed significant increases in the follicular fluid concentrations of estradiol without a change in the follicular fluid concentrations of IGF-I (55,56) and, conversely, significant increases in follicular fluid IGF-I without a change in follicular fluid estradiol (57). Likewise, significant decreases in follicular fluid IGF-I occurred without a change in follicular fluid estradiol of beef heifers (58). Furthermore, concentrations of IGF-I and estradiol in the follicular fluid of cattle can be either correlated negatively (55) or positively (57,59),

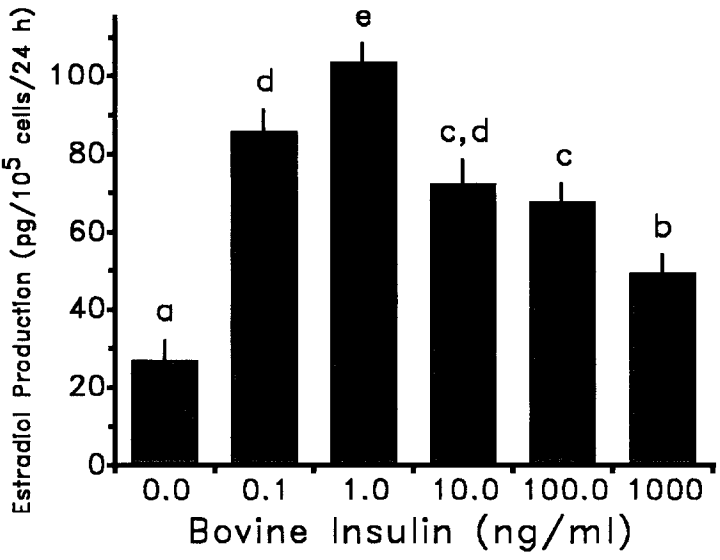

Figure 1. Dose response of insulin on FSH-induced estradiol production by granulosa cells collected from small (1 to 5 mm) bovine follicles. Granulosa cells were cultured for 2 d in the presence of 10% fetal calf serum, washed, and then treated with serum-free medium containing 50 ng/ml ovine FSH and 1 μg/ml testosterone with or without the various doses of insulin for an additional 24 hr. Values are means from three separate experiments. Means with different superscripts differ ($P < 0.05$). Reproduced from Spicer et al. (20) with permission.

suggesting that changes in the intrafollicular concentrations of IGF-I are not consistently regulating estradiol production by bovine follicles. In further support of a negative effect of IGF-I on estradiol production, dairy heifers treated with bST for 21 days had 70% higher plasma IGF-I and 30% lower plasma estradiol concentrations (60). It should be pointed out that, in vivo, coincident changes in ovarian IGFBP levels may influence IGF-I action (see "Ovarian IGFBPs" for further discussion) and that changes in thecal androgen production may influence estradiol production by the intact follicle.

In contrast to studies in cattle, estradiol production by porcine granulosa cells is not altered (19) or is actually inhibited (61) by insulin in vitro, whereas estradiol production by porcine granulosa cells is stimulated by IGF-I (19,62) and to a lesser extent by IGF-II (34). In further support of a role for IGF-I in stimulating follicular estradiol production in pigs, Samaras et al. (63) reported that levels of follicular wall IGF-I mRNA and follicular fluid estradiol were correlated positively ($r = 0.69$), whereas follicular IGF-II mRNA was not significantly correlated with estradiol. Also consistent with these findings, diabetic gilts had significantly lower IGF-I and estradiol concentrations in follicular fluid (64,65), whereas insulin treatment had no effect on follicular fluid estradiol concentrations in PMSG-treated prepubertal gilts (19). Reasons for these apparent differences between cattle and pigs will require further elucidation.

Collectively, in vitro and in vivo data in cattle support the notion that insulin is a better stimulator of granulosa cell estradiol production than is IGF-I, whereas in pigs, IGF-I is a better stimulator of granulosa cell estradiol production than is insulin. Moreover, the lower potency of insulin than IGF-I in studies with rat and primate granulosa cells implies that insulin, with blood levels of 0.2 to 10 ng/ml, may not be a physiologically relevant promoter of estradiol production in those species.

Progesterone production. Numerous studies in several mammalian species including cattle and pigs have shown that insulin, IGF-I, and IGF-II consistently stimulate proges-

terone production by granulosa cells in a rank order potency of IGF-I > IGF-II > insulin (15–17,30,51,66–71). Table 3 summarizes the in vitro effect of IGF-I on progesterone production by ovarian cells of domestic animals. In general, ≥4 d of treatment with IGF-I results in a greater-fold increase in progesterone production than <4 d of treatment (Table 3). However, in those studies that did not correct for final cell numbers, some of this increase is likely due to increased cell proliferation (Table 3). Average ED_{50} values for IGF-I, IGF-II, and insulin are approximately 10, 20, and 100 ng/ml, respectively. These results indicate, like those for estradiol production by rat and human granulosa cells, that the effect of IGF-II and insulin in all mammals studied is mediated at least in part via the type I IGF receptor and that, under normal conditions, insulin may not be a physiologically relevant promoter of progesterone production. In cattle and sheep, concomitant FSH treatment enhances the stimulatory effect of IGF-I on progesterone production (15,30; Table 3). In sheep, it appears that granulosa cells from large (5 to 7 mm) follicles are more responsive to IGF-I in terms of progesterone production than are cells from small (1 to 3 mm) follicles (30,31). Interestingly, IGF-I has no effect on progesterone production by hen granulosa cells (72). We find that, in vivo, concentrations of follicular fluid IGF-I and progesterone are correlated positively in postpartum anestrous cows (55) but not significantly correlated in proestrous cows and heifers (57,59,73). In cyclic gilts, concentrations of follicular wall IGF-I mRNA and follicular fluid progesterone are correlated positively (r = 0.52; 63). In addition, it appears that insulin and IGF-I can stimulate progesterone production by bovine (74,75), porcine (76,77), and rat (78,79) thecal cells. Importantly, IGF-I stimulates the synthesis of cholesterol side-chain cleavage enzyme and its mRNA in porcine granulosa cells (80,81) and rat thecal cells (78,79,82), suggesting an effect of IGF-I on gene expression. In summary, it appears that both insulin and IGF-I can stimulate progesterone production by the granulosa cells of sheep, cattle, and pigs (Table 3). However, the effect of insulin appears to be mediated via the Type I IGF receptor.

TABLE 3. CHRONOLOGIC SUMMARY OF THE STIMULATORY EFFECT OF IGF-I ON PROGESTERONE PRODUCTION (EXPRESSED AS FOLD OF CONTROLS) IN BOVINE, OVINE, AND PORCINE OVARIAN CELLS IN VITRO.

Authors	Ref. No.	Year	Fold of Controls	Co-Trt[a]	Cell Type	Size[b] (mm)	Duration of Trt	Species
Baranao and Hammond	16	1984	>15[c]	None	Granulosa	1–3	4 d	Porcine
Veldhuis et al.	17	1985	8[d]	None	Granulosa	1–5	2 d	Porcine
Veldhuis et al.	66	1986	23[d]	None	Granulosa	1–5	4 d	Porcine
			10[d]	E2	Granulosa	1–5	4 d	Porcine
Schams et al.	68	1988	>14[d]	None	Granulosa	8–15	5 d	Bovine
			<1[d]	None	Granulosa	8–15	2 d	Bovine
Holtorf et al.	69	1989	7[d]	None	Granulosa	>4	5 d	Bovine
Caubo et al.	76	1989	4[d]	None	Theca	8–10	3 d	Porcine
			7[d]	hCG	Theca	8–10	3 d	Porcine
McArdle and Holtorf	83	1989	≤3[d]	None	Luteal		3 d	Bovine
McArdle et al.	71	1991	3[d]	hCG	Granulosa	8–15	4 d	Bovine
			15[d]	None	Granulosa	8–15	4 d	Bovine
Monniaux and Pisselet	30	1992	15[c]	None	Granulosa	8–15	4 d	Ovine
			20[c]	FSH	Granulosa	8–15	4 d	Ovine
Sauerwein et al.	85	1992	<2[d]	None	Luteal		0.5 hr	Bovine
Spicer et al.	15	1993	3[c]	None	Granulosa	1–5	2 d	Bovine
			5[c]	FSH	Granulosa	1–5	2 d	Bovine
Monniaux et al.	31	1994	5[c]	FSH	Granulosa	5–7	4 d	Ovine
			1.5[c]	FSH	Granulosa	1–3	4 d	Ovine

[a] Co-Trt = co-treatment with IGF-I; E2 = estradiol.
[b] Size of follicle from which cells were collected.
[c] Progesterone production corrected for cell numbers at termination of treatments.
[d] Progesterone production (usually, in nanograms per milliliter) not corrected for cell numbers after treatments.

In vitro studies have shown that insulin and IGF-I can stimulate progesterone production by cells from the corpus luteum of cattle, rats, and rabbits in a rank order potency indicative of a Type I IGF receptor effect (83–87). In vivo, we find that serum progesterone and IGF-I concentrations are correlated positively in postpartum dairy cattle (88,89), and that cows in negative energy balance have lower serum IGF-I and luteal phase progesterone secretion than do cows in positive energy balance (88). Furthermore, bST treatment that increases serum IGF-I concentrations during a superovulation paradigm significantly increases serum progesterone concentrations (43). However, bST treatment that increases serum IGF-I during a normal estrous cycle has no effect on serum progesterone (42,60), implying that systemic IGF-I may influence luteal function only under certain conditions.

Androgen production. Studies indicate that both insulin (ED_{50} = 200 ng/ml) and IGF-I (ED_{50} = 10 ng/ml) have stimulatory effects on LH-induced thecal cell androgen biosynthesis in pigs (76,77), rats (78,79,90–92), and humans (93). The mechanism of action of IGF-I on LH-stimulated androgen and progesterone production by rat thecal cells appears to be via the stimulation of thecal cell 3β-hydroxysteroid dehydrogenase mRNA (78), cholesterol side chain cleavage mRNA, and 17_{α}-hydroxylase mRNA (79,82). A study with thecal cells isolated from 12 to 15-mm bovine follicles indicated that 2 g/ml of insulin or 100 ng/ml of IGF-I had no effect on basal androstenedione production (54). Similarly, we find that insulin and IGF-I, at concentrations found in the blood of cattle, have no effect on basal androstenedione production but both enhanced LH-induced androstenedione production by two- to sixfold in thecal cells from ≥8-mm bovine follicles (75; Figure 2). However, further research is needed to determine the mechanism of action of IGF-I and insulin in bovine thecal cells. In summary, it appears that, in all species studied to date, insulin and IGF-I synergize with LH to promote thecal cell androgen production and that bovine thecal cells appear much more sensitive to insulin than do porcine or rat thecal cells.

Ovarian insulin and IGFs. The presence of insulin and IGFs in the ovary was first reported by Hammond et al. (94,95) in the pig. Since that time, the presence of IGFs in the follicular fluid of several other species has been reported, including cattle, sheep, horses, and humans (40,41,55,56,58,73,96–98). In general, concentrations of insulin (0.5 to 10 ng/ml), IGF-I (100 to 500 ng/ml), and IGF-II (20 to 1,000 ng/ml) are equal to or lower in follicular fluid than in plasma (57,96,99,100). However, after a 2-day fast in heifers, follicular fluid concentrations of IGF-I are higher than plasma concentrations (73). Similarly, in fasted humans, follicular fluid concentrations of insulin are higher than plasma concentrations (101). Thus, it appears that nutritional status influences the relationship between ovarian and systemic levels of insulin and IGFs.

In studies with cattle, pigs, and sheep, we and others have found that small (≤4 mm) antral follicles have lower concentrations of IGF-I than do large (≥ 5 mm) antral follicles (40,41,57,59,99), and concentrations of IGF-I in follicular fluid are correlated positively (r = 0.3 to 0.7) with follicular diameter in cattle (55,57,59). Similarly, IGF-I mRNA levels in the follicular walls of cyclic gilts were correlated (r = 0.59) with follicular diameter (63). In contrast, we find slightly higher levels of IGF-II in small vs. large bovine (Figure 3) and ovine follicles (98), but no difference between small and medium porcine follicles (40). Whether these differences in concentrations of IGFs between small and large follicles are the result of differences in local biosynthesis or changes in the diffusion of IGFs from plasma remains to be determined. Nonetheless, IGF-I and IGF-II are most likely produced by the ovary. Numerous studies have reported the presence of IGF-I and IGF-II mRNA in ovarian tissue of rats, women, cattle, pigs, and sheep including granulosa, thecal, stromal, and luteal cells (15,50,98,100,102–108). Rat gran-

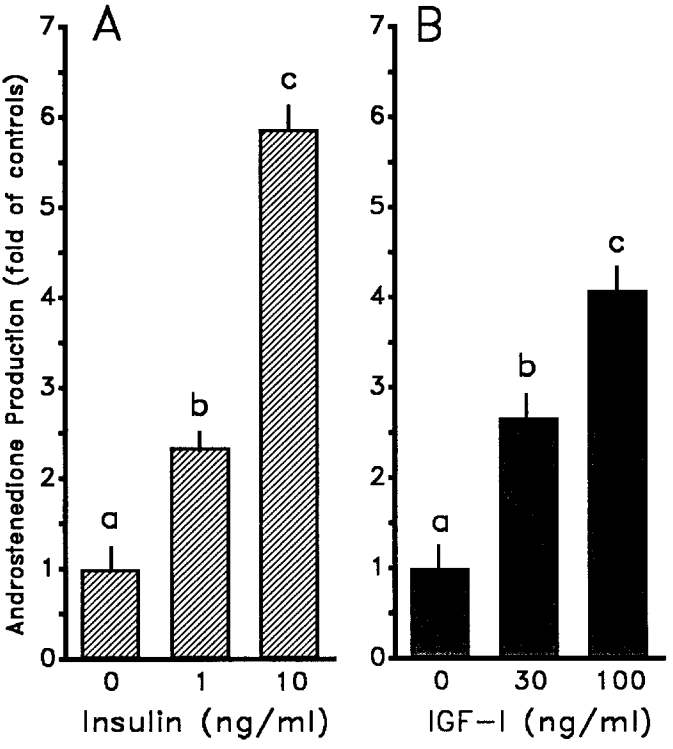

Figure 2. Effects of insulin and IGF-I on LH-induced androstenedione production by thecal cells collected from large (≥8 mm) bovine follicles. Thecal cells were cultured for 2 d in the presence of 10% fetal calf serum, washed, and then treated with serum-free medium containing 100 ng/ml bovine LH with or without the various doses of insulin (A) or IGF-I (B) for an additional 48 hr. Values are means from three separate experiments and are expressed as fold of controls, which averaged 54 ± 14 and 117 ± 26 pg/10^5 cells per 24 hr for Panels A and B, respectively. Means with different superscripts differ ($P < 0.05$). Data are modified from Stewart et al. (75).

ulosa cells appear to contain exclusively IGF-I mRNA, whereas rat thecal cells contain exclusively IGF-II mRNA (105,106). We find that both granulosa and thecal cells of bovine follicles contain IGF-I mRNA (15; Figure 4). Recent studies indicate that IGF-II mRNA exists in porcine ovaries (100) as well as in ovine follicular walls (98). In addition, porcine and bovine granulosa cells secrete IGF-I (15,22,23; Table 4), whereas human granulosa cells predominantly secrete IGF-II (109), exclusively localize IGF-II, and contain IGF-II mRNA (50,110,111). In sheep, more follicular walls have detectable IGF-II mRNA than IGF-I mRNA, as assessed by reverse transcriptase–polymerase chain reaction (98). Also, follicular fluid IGF-II concentrations vary with genotype, whereas IGF-I does not (98).

Some studies have indicated that ST and gonadotropins can stimulate IGF-I and(or) IGF-II production in porcine (22,23; Table 4) and human (109) granulosa cells, respectively. Also, the in vitro perfusion of intact rabbit ovaries with bST (≥10 ng/ml) for >4 hr stimulated IGF-I secretion coincident with an increase in follicular diameter (112). In contrast, the production of IGF-I by bovine granulosa cells in vitro was unaltered by treatment with ST and FSH but was inhibited by treatment with insulin (15; Table 4). Moreover, we are unable to detect IGF-II production by cultured bovine granulosa cells (Spicer and Vernon, unpublished observations). In vivo, 20- to 40-d treatment of pST in prepubertal gilts increased serum and follicular fluid IGF-I concentrations but de-

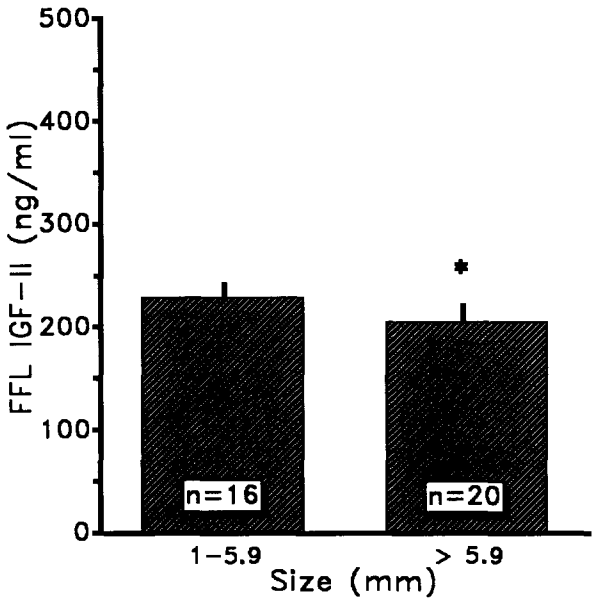

Figure 3. Concentrations of IGF-II in follicular fluid (FFL) of small (1 to 5.9 mm) or large (≥6.0 mm) follicles collected from heifers during the preovulatory period. Values were determined by a radioimmunoassay as described in Spicer et al. (98). Asterisk indicates mean differs from 1 to 5.9 mm mean ($P < 0.05$).

creased or had no effect on serum and(or) follicular fluid IGF-II concentrations (100). The treatment of prepubertal gilts with PMSG for 3 d has been shown to either increase (99) or not affect (100) follicular fluid IGF-I concentrations. The relative contribution of changing systemic IGF levels to those in follicular fluid in these and other studies remains to be elucidated. Whether species and(or) methodologic differences exist in terms of hormone-regulated IGF production by granulosa cells also will require further study.

In summary, it appears that, regardless of species, ovarian IGF-I production and IGF-II production are under different control mechanisms and that concentrations of IGF-I increase as follicles develop. However, species differences may exist in terms of the specific cell layer(s) within the follicle that may produce IGF-I and IGF-II and in terms of the hormones that regulate their production.

Ovarian insulin and IGF receptors. Specific receptors for insulin and IGFs and their mRNA exist in various types of ovarian cells including granulosa cells (16,18,20,21, 51,71,110,111,113), thecal cells (90,91,93,110,111,114), luteal cells (84,85,87,114–116), and ovarian stromal cells (117–119). Affinity constants for the high-affinity binding sites of insulin, IGF-I, and IGF-II in ovarian tissues range from 1 to 5 nM (Table 5).

Whether numbers of insulin and IGF receptors change as follicles develop is unclear. In swine, granulosa cells from small follicles have fewer insulin receptors than do cells from medium or large follicles (18). Similarly, in women, granulosa and thecal cells from dominant follicles localize more insulin receptor mRNA than do cells from small antral follicles (110,111). However, numbers of total ovarian insulin receptors do not change during the menstrual cycle in women (117). In comparison to insulin receptors, the number of granulosa cell IGF-I receptors is similar among small, medium, and large porcine follicles (19,120). In contrast, we have observed that granulosa cells from large bovine follicles, cultured in vitro, have a 15-fold higher number of IGF-I receptors than

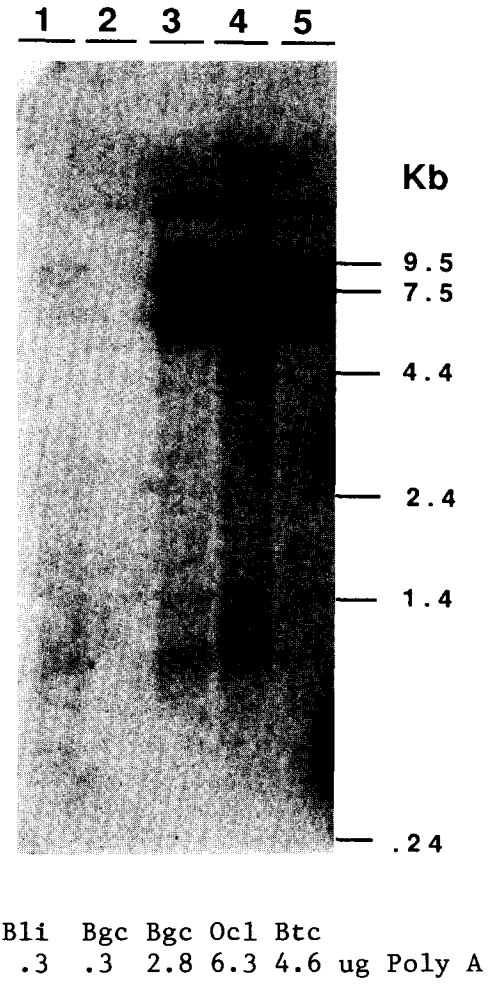

Figure 4. A representative northern blot of ovarian IGF-I mRNA in granulosa, thecal, and luteal tissue of cattle and(or) sheep. Granulosa, thecal, and luteal RNA was subjected to electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled IGF-I cDNA as previously described by Spicer et al. (15), except that a bovine cDNA (182) was used instead of a human cDNA. Bli = bovine liver; Bgc = bovine granulosa cells; Btc = bovine thecal cells; Ocl = ovine corpora lutea (Day 11 of estrous cycle); Kb = kilobases.

do cells from small follicles (21). A recent report using the cellular localization of [^{125}I]IGF-I within ovaries from bovine fetuses and neonatal calves lends support to our studies in that specific IGF-I binding increased as follicles enlarged from preantral to antral size, concomitant with increases in FSH and LH binding (121). Atretic follicles from these ovaries contained significantly lower numbers of specific IGF-I-binding sites than did nonatretic follicles (121). A similar study using in situ hybridization histochemistry in rat ovaries indicated that IGF-I mRNA is lost whereas IGF-I receptor mRNA is increased as the granulosa cells differentiate and luteinize, whereas IGF-I receptor mRNA disappeared from the granulosa cells of atretic follicles (122). These results are in contrast to the aforementioned studies in pigs in which the number of IGF-I receptors in granulosa cells did not differ between small, medium, and large follicles (19,120) when freshly isolated granulosa cells were used. Such cell populations from freshly isolated follicles are a composite of both live and dead cells from both healthy and atretic follicles (123,124).

TABLE 4. THE OVARIAN GRANULOSA CELL IGF SYSTEM: REGULATION BY LOCAL AND SYSTEMIC FACTORS IN SWINE AND CATTLE.

IGF Component	Animal	Regulation[a]		
		Stimulators	No Effect	Inhibitors
GC IGF-I production[b]	Swine	LH, ST, FSH EGF/TGFα, PDGF	PRL, bFGF	TGFβ
	Cattle	?[c]	ST, FSH	Insulin
GC IGFBP-2 production	Swine	IGF-I, Insulin	IGF-II, EGF, E2	TGFβ, FSH
	Cattle	?	?	?
GC IGFBP-3 production	Swine	IGF-I, Insulin, $PGF_{2\alpha}$	IGF-II, EGF, E2	TGFβ, FSH, PGE_2
	Cattle	?	?	?
GC IGFBP-4 mRNA	Swine	?	FSH, IGF-I	?
	Cattle	?	?	?
GC IGFBP-5 mRNA	Swine	IGF-I	FSH	?
	Cattle	?	?	?
GC IGF-I receptors	Swine	E2, corticoids	?	?
	Cattle	E2, EGF, FSH	LH, PROG	bFGF
GC IGFBP protease	Swine	FSH	Insulin, PGE_2, ST PRL, E2, PROG, TGFβ	IGF-I, IGF-II
	Cattle	?	?	?

[a] Abbreviations not defined in text: PRL = prolactin; TGF = transforming growth factor; E2 = estradiol; PROG = progesterone; PDGF = platelet-derived growth factor.
[b] GC = granulosa cell.
[c] ? = not yet investigated.

Thus, the presence of dead cells would likely mask any real "size" effect on the number of IGF-I receptors. In further support of the notion that the number of IGF-I receptors in granulosa cells is higher in large vs. small follicles, others (30,48) have shown that steroid production by granulosa cells from large follicles is dramatically increased by IGF-I whereas IGF-I has little or no effect on steroid production by granulosa cells from small follicles. In ovine follicles, histochemically localized [^{125}I]IGF-I did not differ between small (<2 mm) and large (>2 mm) follicles (125), but this may have been due to a concomitant decrease in IGFBPs (see next section). Recent studies using the cellular localization of IGF-I receptor mRNA in human ovaries indicate that IGF-I receptors are present in granulosa cells of primary and antral follicles of infant ovaries and graafian follicles of adult ovaries (110,111,119,126). Interestingly, human oocytes intensively localize IGF-I receptor (127) and its mRNA (126) whereas rat oocytes localize very little IGF-I receptor mRNA (122). Collectively, ours and other recent studies provide evidence that IGF-I receptors are present in granulosa cells throughout ovarian follicular development, increase as small antral follicles develop into graafian follicles (i.e., as granulosa cells differentiate), and decrease during atresia.

The hormonal regulation of granulosa cell IGF-I receptors is not completely understood. We have observed that numbers of IGF-I receptors in granulosa cells of small

TABLE 5. AFFINITY CONSTANTS AND CROSS-REACTIVITY OF IGF-I, IGF-II, AND INSULIN RECEPTORS IN OVARIAN TISSUE.[a]

Receptor Type	Kd[b] (nM)	Cross-reactivity (%)		
		IGF-I	IGF-II	Insulin
IGF-I	1–3	100	10	0.3
IGF-II	1–5	0.5	100	0.0
Insulin	1–2	1	4	100

[a] Compiled from numerous studies using ovarian tissues.
[b] Dissociation constant of the hormone-receptor interaction.

bovine follicles are increased by EGF, estradiol, and FSH, decreased by basic fibroblast growth factor (bFGF), and unaffected by LH and progesterone (21; Table 4). In contrast, numbers of IGF-I receptors in granulosa cells of large bovine follicles were unaffected by EGF, estradiol, FSH, bFGF, or LH (21). As summarized in Table 4, the number of granulosa cell IGF-I receptors is increased by estradiol (66) and dexamethasone (128) in porcine granulosa cells and by FSH, LH, and β-adrenergic agonists in rat granulosa cells (129,130). The stimulatory effect of EGF, estradiol, and FSH on IGF-I receptors in granulosa cells of small follicles may explain why these hormones synergize with IGF-I to enhance granulosa cell mitogenesis and(or) differentiation. In contrast to IGF-I receptors, IGF-II receptor mRNA and protein are localized within both granulosa and thecal cells of human antral follicles (110,111). In sheep, IGF-II receptors appear to be primarily present in granulosa cells of atretic follicles and in thecal cells of healthy follicles, although specific [^{125}I] IGF-II binding was also detected in oocytes, stroma, and perivascular cells (131). Determination of whether oocytes of domestic animals contain IGF-I receptors or whether follicles of pigs and cattle contain IGF-II receptors awaits further study.

In rats, large luteal cells have more IGF-I-binding sites than do small luteal cells, and luteal IGF-I receptor mRNA decreases between Days 7 and 15 of pregnancy (87). Whether these changes in luteal IGF-I receptors occur during pregnancy in domestic animals remains to be determined. However, numbers of luteal IGF-I, IGF-II, and insulin receptors do not differ between Days 5 to 7, 8 to 12, or 15 to 18 of the estrous cycle in cattle (85). Insulin receptors are also present in rat luteal cells (84). Additional studies are needed to determine if insulin receptor mRNA or numbers of insulin receptors change during the luteal development of other domestic animals.

In summary, most cell types within the ovary contain insulin and IGF receptors, regardless of species. Limited studies indicate that numbers of IGF-I receptors in granulosa cells are hormonally regulated, increase as the follicle develops, and decrease during atresia. However, the hormonal control of IGF-I receptors in thecal and luteal cells awaits further study. Determination of the regulation of ovarian cell insulin receptor and its mRNA also awaits study.

Ovarian IGFBPs. The IGFBPs are high-affinity carrier proteins that function as transport proteins for the IGFs in the systemic circulation. The IGFBPs prolong the half-life of IGFs as well as serve to partition IGFs in extracellular fluids (for reviews, see Refs. 132–134). To date, six unique species of IGFBPs have been identified: IGFBP-1, -2, -3, -4, -5, and -6 (Table 6). The reader is referred to the aforementioned reviews for further details of the chemistry of these IGFBPs. In general, there is about a 50% amino acid homology among the IGFBPs within species and about an 80% amino acid homology for each IGFBP across species. The liver produces IGFBPs along with numerous other tissues including the ovary. Since 1989, when the structure of an IGFBP and its mRNA in ovarian tissue were first reported (135–137), research has implicated the IGFBPs as potential regulators of follicular development. In particular, the IGFBPs in the ovary appear to play an important role in regulating the biologic activity of IGF-I (12,13,137–139). For example, IGFBP-2, -3, -4, and -5 inhibit FSH-induced estradiol synthesis in cultured rat and human granulosa cells by sequestering IGF-I (137,140,141), and the intrabursal administration of IGFBP-3 reduces the number of human chorionic gonadotropin (hCG)–induced ovulations in immature rats (138). Consistent with the idea that IGFBPs inhibit follicular steroidogenesis, levels of follicular fluid progesterone are negatively correlated ($r = -0.70$) with follicular wall IGFBP-2 mRNA (63). Also, follicular fluid estradiol is negatively correlated with follicular fluid IGFBP-2 in ovine follicles ($r = -0.74$; 98) and bovine follicles ($r = -0.57$; 142).

TABLE 6. SUMMARY OF THE SPECIFIC TYPES OF IGFBPs.

IGFBP-1:	25–34 kDa, nonglycosylated (fetal serum, amniotic fluid, endometrium)[a]
	*Produced by human but not by porcine or rat granulosa cells
	*Affinity: IGF-I = IGF-II
IGFBP-2:	29–40 kDa, nonglycosylated (fetal serum, CSF[b])
	*Increases with fasting, decreases with ST treatment
	*Produced by porcine and rat granulosa cells, and by human granulosa and thecal cells
	*Affinity: IGF-II > IGF-I
IGFBP-3:	150 kDa, glycosylated composed of two subunits, 28 to 53 kDa (adult serum)
	*ST dependent, decreases with fasting
	*Produced by luteinized pig granulosa cells, rat corpora lutea, and by human granulosa and thecal cells
	*Affinity: IGF-I = IGF-II
IGFBP-4:	24–30 kDa (rat serum)
	*Localized in rat atretic antral and preantral follicles and by human granulosa and thecal cells
	*Affinity: IGF-I = IGF-II
IGFBP-5:	29–31 kDa (rat serum)
	*Localized in rat atretic antral and preantral follicles, and corpora lutea, and by human granulosa, thecal and ovarian stromal cells
	*Affinity: IGF-II > IGF-I
IGFBP-6:	21–32 kDa (adult serum, CSF)
	*Localized in rat thecal cells; nondetectable in human granulosa and thecal cells
	*Affinity: IGF-II ≫ IGF-I

[a] Location where IGFBP was first identified.
[b] CSF = cerebrospinal fluid.

Follicular fluid levels of IGFBP-2 as well as other lower molecular weight IGFBPs (but not IGFBP-3), assessed by ligand blotting, decrease as follicles enlarge in sheep (139), pigs (41,143,144), and cattle (142). Similarly, we (98) have observed significantly lower total IGFBP activity and three forms of IGFBPs (IGFBP-2, 27 to 29 and 24 kDa) in large follicles vs. small follicles of ewes 48 hr after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection and have found that the genotype of ewe had no effect on total IGFBP activity in follicular fluid (as assessed by both exchange assay and ligand blot). The 30% lower total IGFBP activity in large vs. small follicles was not a result of a decrease in IGFBP-3 but rather of a decrease in other lower molecular weight IGFBPs, as previously reported for pigs and cattle (63,142–144). In cyclic gilts, levels of follicular wall IGFBP-2 mRNA and follicular diameter were correlated negatively ($r = -0.70$; 63). Similarly, follicular fluid IGFBP-2 and follicular diameter were correlated negatively in cattle ($r = -0.81$; 142). A decrease in intrafollicular IGFBP activity together with an increase in IGF-I concentrations as follicles develop is thought to provide a coordinated sequence of events that facilitates IGF-I bioavailability for the support of follicular growth and granulosa cell differentiation (12,13). Other data indicate that atresia in ovine follicles <2 mm in diameter is associated with an increase in IGFBP-like activity localized within the granulosa cells and follicular antrum (125). Similarly, atretic follicles in sheep (139), cattle (142), and women (145) contain more IGFBP-2 activity than do healthy follicles. The cause of these changes in follicular fluid IGFBP activity as follicles develop and become atretic is unknown but may involve specific, hormonally regulated proteases that degrade specific IGFBPs (146,147).

Studies evaluating the cellular source of the various ovarian IGFBPs indicate that porcine granulosa, thecal, and(or) luteal cells appear to produce IGFBP-2 and -3 (25,26,147–149). However, in follicular walls (granulosa plus theca) of cyclic gilts, IGFBP-2 mRNA but not IGFBP-3 mRNA could be detected by northern analysis (63). Porcine granulosa cells also appear to produce IGFBP-4 and -5 (150). In comparison, rat granulosa cells appear to produce IGFBP-2, -4, -5, and -6 (151–154). Rat thecal and luteal cells appear to produce only IGFBP-3 (151,155,156). IGFBP-1, produced by the human ovary (13,157), is not found in the porcine or rat ovary. At present, it is uncertain

whether the 29-kDa IGFBP found in the follicular fluid of sheep and cattle is IGFBP-1 or some other IGFBP. Recently, a 31- to 32-kDa IGFBP in bovine follicular fluid has been tentatively identified as IGFBP-5 (158) by western blotting. Verification of these possibilities will require the development of antibodies specific to each of the IGFBPs in each species under investigation. In general, the activity of IGFBP-2 in follicular fluid is greater than or equal to the activity found in plasma, whereas IGFBP-3 activity is lower in follicular fluid than in plasma (98,158–160). How follicular fluid IGFBP-4 and IGFBP-6 activity compares to that found in plasma is unclear because, as mentioned, the specific antibodies necessary to identify them on western blots have yet to be developed for domestic animals. Because of the relatively high IGFBP activity in plasma, it is likely that changing follicular permeability to plasma IGFBPs, in addition to local production, may explain some changes in follicular fluid IGFBP activity as well as alter the bioavailability of intrafollicular IGF-I and -II. Serum IGFBP-2 and IGFBP-3 as well as IGF-I change with feed restriction (161,162), and thus, nutritional state may influence ovarian IGFBP activity. However, like IGF-I and IGF-II, the relative contribution of serum-derived IGFBPs to follicular fluid IGFBP activity remains to be determined.

Additional in vivo evidence indicates that total amounts of whole ovarian IGFBP-2 mRNA do not change from Day 11 to 21 of the estrous cycle in gilts, whereas amounts of ovarian IGFBP-3 mRNA decrease during this same interval (149). Interestingly, amounts of IGFBP-2 mRNA are lower whereas amounts of IGFBP-3 mRNA are higher in luteal tissue than in thecal or granulosa cells in gilts (149). Furthermore, amounts of IGFBP-2 increase whereas amounts of IGFBP-3 decrease in the fluid of preovulatory follicles after hCG injection in sows (144). It is speculated by Grimes et al. (148) that the porcine granulosa cells used for their studies luteinize in culture. This may explain why IGFBP-3 is produced by cultured porcine granulosa cells. Thus, together with results from cell culture studies, it appears that IGFBP-2 production decreases and IGFBP-3 production increases as granulosa cells differentiate and luteinize, at least in swine. Additional studies are required to determine if these changes occur in sheep and cattle.

The identification of factors that regulate the production of ovarian IGFBPs has been an active area of research in recent years (Table 4). Of the hormones evaluated, FSH (200 ng/ml) is one of the most potent inhibitors of IGFBP-2 and -3 activity produced by porcine granulosa cells (24). Similarly, in human granulosa cells, IGFBP-2 production is inhibited by hCG and dibutyryl cAMP (163). In cultured rat granulosa cells, total IGFBP activity in spent media was also reduced after FSH ($\geq$30 ng/ml) treatment (164,165). The administration of exogenous FSH to cyclic cows decreased the amounts of IGFBP-2 and lower molecular weight IGFBPs in large ($\geq$8 mm) estrogen-active follicles but had no effect on these IGFBPs in large estrogen-inactive follicles or on IGFBP-3 in either type of large follicle (142). Prostaglandin E_2 (PGE_2) and dibutyryl cAMP have also been shown to inhibit IGFBP activity in cultured porcine (148) and murine granulosa cells (166), suggesting that any cAMP-induced intracellular cascade may inhibit granulosa cell IGFBP activity. Interestingly, the production of IGFBP-1 by human granulosa-luteal cells is stimulated by PGE_2 (157) but inhibited by FSH (167). How gonadotropin-induced cAMP production reduces IGFBP activity is unclear, but recent evidence in rats and pigs suggests that it may involve the induction of specific IGFBP proteases (146,147; Table 4). Further research will be required to determine if other domestic animals have specific ovarian IGFBP proteases.

In contrast to FSH, IGF-I and insulin have been shown to stimulate IGFBP-2 and -3 activity in cultured porcine granulosa cells (25,62) whereas estradiol, EGF, and transforming growth factor-β had no significant effect on IGFBP-2 or -3 activity when corrected for changes in cell numbers (24). However, diethylstilbestrol (DES) treatment in

hypophysectomized rats increased ovarian IGFBP-2 mRNA levels yet decreased ovarian IGFBP-6 mRNA levels (152,168). In prepubertal pigs, 20 to 40 d of daily pST injections increased follicular fluid IGFBP-3 activity but did not affect follicular fluid IGFBP-2 activity or whole ovarian IGFBP-2 mRNA, whereas whole ovarian IGFBP-3 mRNA levels decreased significantly (100). These effects of pST were completely eliminated 3 d after gilts were treated with PMSG (100). In contrast, sustained-release implants of pST for 41 d increased IGFBP-2 activity in the follicular fluid of small follicles, had no effect on IGFBP-2 activity in medium follicles, and decreased serum IGFBP-2 activity in prepubertal gilts (41). Thus, the mode of ST treatment may influence the ovarian IGFBP response to ST. Alone, PMSG decreased follicular fluid IGFBP-2 activity but had no effect on the amount of whole ovarian IGFBP-2 or -3 mRNA or on follicular fluid IGFBP-3 activity (100). Why amounts of whole ovarian IGFBP-2 and -3 mRNA do not change in concert with follicular fluid concentrations of IGFBP-2 and -3 is unclear but likely reflects the heterogeneous cell population of whole ovaries as well as the multiple sources of intrafollicular IGFBPs. A comprehensive scheme of how these various hormones and growth factors interplay to regulate ovarian IGFBP activity and gene expression in vivo has yet to be devised.

In summary, IGFBP activity in the follicular fluid of pigs, sheep, and cattle decreases as follicles develop and become estrogen active. As follicles undergo atresia and become estrogen inactive, IGFBP activity increases. These changes in IGFBP activity are due to changes in IGFBP-2 and other lower molecular weight IGFBPs and not to changes in IGFBP-3. The hormones that regulate these changes in IGFBP activity are not well characterized for sheep and cattle but, on the basis of studies in rats and pigs, may involve FSH, insulin, and IGF-I (Table 4). The decrease in IGFBP activity together with an increase in IGF-I concentrations as follicles develop likely provides a coordinated sequence of events that facilitates IGF-I and -II bioavailability for the support of follicular growth and increased steroidogenesis by granulosa cells. During luteinization, at least in pigs, IGFBP-3 activity and gene expression increase.

CONCLUSIONS AND FUTURE DIRECTIONS

Evidence accumulated from in vitro studies over the past 10 years clearly indicates that IGF-I at physiologic levels stimulates granulosa cell mitogenesis and steroidogenesis in most species studied. These effects appear to be mediated by specific IGF-I receptors in granulosa cells that increase as follicles develop. Whether IGF-I stimulated mitosis is the reason that follicular diameter and follicular fluid IGF-I are correlated positively in vivo remains to be determined. Additional in vivo studies have provided evidence that systemic and(or) ovarian IGF-I may be involved in multiple ovulations in cattle (57) but not in sheep (98,169). Comparable data for IGF-II are not available and should be the focus of further research. Strong evidence also exists for a role of insulin and IGF-I in luteal function. Although insulin has ovarian effects that are similar to those of the IGFs, the ED_{50} of insulin implies that its effects are not physiologically relevant except in cattle. Recent studies in our laboratory indicate that insulin at physiologic levels indeed may affect ovarian function in cattle.

Both IGF-I and IGF-II are produced by ovarian cells and thus may contribute to an autocrine or paracrine system designed to enhance follicular growth and gonadotropin-induced differentiation. Studies using the in situ hybridization of IGF-I and IGF-II mRNAs and insulin, IGF-I, and IGF-II receptor mRNAs will likely reveal the intricacies of the insulin and IGF system that otherwise could not be ascertained. For example, preliminary studies using in situ hybridization in ewes indicate that IGF-I mRNA is mainly localized in granulosa, thecal, and luteal cells but the intensity of staining does not

change with follicular or luteal development (170). Elegant studies using coincident in situ hybridization of mRNAs and immunocytochemical localization of proteins involved in the IGF system have been conducted with human ovaries (111) and should be a focus of research in domestic animals in the future.

Adding to the complexity of the regulatory role for IGFs is the presence of as many as six specific forms of IGFBPs within the ovary. These IGFBPs are produced by ovarian cells and their production is hormonally regulated. Species differences exist with regard to the specific IGFBP produced and the cell type that produces it, and further research is needed to clarify these differences. A decrease in intrafollicular IGFBP activity together with an increase in IGF-I as follicles develop may facilitate IGF-I bioavailability for the support of follicular growth and for granulosa and thecal cell differentiation. Recent studies indicate that specific proteases may alter porcine (150) and rat (146) ovarian cell IGFBP activity, but more research is required in this area. Future research also should address the role(s) that IGFs and IGFBPs may play in the growth and selection of the follicle(s) destined for ovulation. We have observed that heifers fed to lose body weight had three waves of follicular growth during an estrous cycle whereas heifers fed to gain body weight had primarily two waves of follicular growth (171).

In close anatomical proximity to the ovary is the uterus. Because the uteri of pigs, cattle, and sheep also produce and respond to IGF-I (172–175), future research should determine if communication between the ovarian and uterine IGF system occurs. Current evidence suggests that ovarian and conceptus steroids (i.e., estrogens) regulate the uterine IGF system in rats and pigs (172,176,177). Although additional research will be required to determine if uterine factors (e.g., $PGF_{2\alpha}$, cytokines) regulate the ovarian IGF system, recent data suggest that $PGF_{2\alpha}$ stimulates IGFBP-3 production by luteinized porcine granulosa cells (148).

Finally, further research is needed in domestic animals to determine if manipulation of the ovarian IGF system may result in improved reproductive efficiency. As mentioned earlier, Gong et al. (43) found that exogenous bST treatment, which increased serum IGF-I concentrations, increased the number of PMSG-induced ovulations in cattle. In addition, Rieger et al. (178) using cattle and Cognie et al. (179) using sheep have shown that bST injections given concomitantly with FSH injections during a normal superovulatory regimen increased the percentage of transferable embryos. In prepubertal gilts, long-term pST treatment increased the number of antral follicles, which was positively correlated with concentrations of IGF-I in serum (40,41); whether this increased number of follicles will improve reproductive efficiency remains to be determined. It should be emphasized that pST treatment in prepubertal gilts can also inhibit reproductive functions. For example, Bryan et al. (180) observed a significant reduction in the LH responsiveness of porcine granulosa cells cultured in vitro from pST-treated gilts. Similarly, we have observed that prepubertal gilts treated with pST have decreased numbers of LH/hCG-binding sites in the granulosa cells of medium follicles (40). In dairy cattle, increased luteal function is associated with increased concentrations of IGF-I in serum during early lactation (88; 181); whether this translates into improved pregnancy rates will require further study.

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